Phytopathologia Mediterranea (2015) 54, 1, 76–85
DOI: 10.14601/Phytopathol_Mediterr-14303

RESEARCH PAPER

Fusarium oxysporum f. sp. *papaveris*: a new forma specialis isolated from iceland poppy (*Papaver nudicaule*)

Giuseppe ORTU[1], Domenico BERTETTI[1], Patrizia MARTINI[2], Maria Lodovica GULLINO[1,3] and Angelo GARIBALDI[1]

[1] *Agroinnova – Centre of Competence for the Innovation in the Agro-Environmental Sector, University of Turin, Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy*
[2] *Istituto Regionale per la Floricoltura, Via Carducci 12, 18038 Sanremo, Italy*
[3] *DISAFA - Department of Agricultural, Forest and Food Sciences, University of Turin, Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy*

Summary. Symptoms of a new vascular wilt were observed on several plants of *Papaver nudicaule* cultivated at the Regional Institute for Floriculture in Sanremo and in a nursery near Ventimiglia, Liguria region, Northern Italy. Morphology and ITS sequence analysis characterize the pathogen as *Fusarium oxysporum*. To identify the *forma specialis*, eight isolates obtained from infected tissues were used for a phylogenetic analysis based on translation elongation factor 1-α (*EF-1α*) gene and four polygalacturonase genes (*Pg1*, *Pg5*, *Pgx1* and *Pgx4*). After amplification by PCR, the gene sequences were aligned with corresponding sequences from other *formae speciales* of *Fusarium oxysporum* obtained from GenBank databases in order to build phylogenetic trees. Results obtained for each genomic region showed that the isolates derived from *P. nudicaule* form a unique group well separated from other *formae speciales*. To our knowledge, this is the first report of *F. oxysporum* on *P. nudicaule* and together with the phylogenetic analysis and positive pathogenicity tests permits us to introduce a new *forma specialis* named *F. oxysporum* f. sp. *papaveris*.

Key words: *Fusarium*, *EF-1α*, endopolygalacturonase, exopolygalacturonase.

Introduction

Papaver nudicaule (Iceland poppy) belongs to the *Papaveraceae* family. It is a herbaceous plant, 40–50 cm tall; in nature it inhabits steppes, stony slopes of mountains and sandy river banks (Preininger, 1986). It is spread in the Mongolian-Siberian area of Central Asia and is grown for the production of cut flowers or in flower beds and borders in public and private gardens (Věžník *et al*., 1987). The plant is perennial, but usually cultivated as an annual, producing showy flowers with large colourful petals that vary between different cultivars. During autumn 2011, several plants cultivated at the Regional Institute for Floriculture in Sanremo and in a nursery near Ventimiglia (Imperia province, Northern Italy) showed symptoms of vascular wilt. The first symptoms consisted of chlorosis and premature drop of leaves. As the disease progressed, the leaves became yellowed and wilted, the stem wilted, bent and eventually rotted starting from the base, without dropping. Brown discolorations were observed in the vascular system. Fifteen to twenty percent of the plants were affected in the commercial nursery (Garibaldi *et al*., 2012).

Eight isolates obtained from infected tissue were characterized microscopically and by analysis of ITS sequence data. The results showed that the pathogen was *Fusarium oxysporum*. The pathogenic ability and virulence of the isolates were confirmed with a pathogenicity assay. All inoculated plants showed typical wilt symptoms 14 days after inoculation (Garibaldi *et al*., 2012).

Corresponding author: G. Ortu
E-mail: giuseppe.ortu@unito.it

ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

Fusarium oxysporum is known as the causal agent of vascular wilt in more than 100 different plant species. Over the years, the pathogenic isolates of *F. oxysporum* have been grouped into different *formae speciales* according to their host (Armstrong and Armstrong, 1981; Garibaldi and Gullino, 1985; Di Pietro *et al*., 2003; Ortu *et al*., 2013). More than 120 different *formae speciales* have been described (Baayen *et al*., 2000; Kirk *et al*., 2008; O'Donnell *et al*., 2009; Leslie, 2012). The identification of pathogenic *F. oxysporum formae speciales* is mainly based on bioassays exploiting the host plant specificity (Recorbet *et al*., 2003). However, molecular tools are becoming increasingly available . The phylogeny of studied taxa is regarded as well-resolved when sequences for a number of genes or regions yield congruent topologies that can be combined and obtain statistical support (Soltis *et al*., 1999; Lutzoni *et al*., 2004).

The specialization of plant pathogens may be due to small nucleotide differences in a specific gene (Joosten *et al*., 1994; Hirano and Arie, 2006). On the other hand, virulence may be attributed either to a single gene or to a set of genes that confer a specific characteristic to the pathogen, such as production of host-specific toxins (Friesen *et al*., 2006; Van der Does and Rep, 2007).

Studies on *F. oxysporum* f. sp. *lycopersici* have shown that the secreted-in-xylem (SIX1) gene, encoding for a small cysteine-rich secreted protein, confers full virulence to the fungus on tomato plants (Rep *et al*., 2004, 2005). In fungal pathogens, polygalacturonase represents the major cell wall-degrading enzyme (CWDE); different studies have demonstrated its suitability for distinguishing populations of a fungus (Schilling *et al*., 1996; Hirano and Arie, 2006).

In this work, four genes were analyzed: two encoding for two endopolygalacturonases and two for two exopolygalacturonases. These genomic regions were used in a phylogenetic analysis to verify the possible presence of a new *forma specialis* of *Fusarium oxysporum.*

Materials and methods

Fungal isolates

Infected tissues of *P. nudicaule* were washed and surface-sterilized for 5 min in 1% (v/v) sodium hypochlorite and rinsed 3 times with sterile distilled water. The tissue was sliced with a sterile knife, and incubated on Komada's *Fusarium*-selective medium at 25°C for 7 days (Komada, 1975). Isolates were transferred to potato dextrose agar (PDA, Oxoid) amended with 0.5 mg/mL streptomycine sulphate, and incubated for 7 days at 25°C. For each isolate obtained, single-spore cultures were established by serial dilution of a conidial suspension on PDA, and single germinated macroconidia were transferred to fresh plates. The isolates recovered from *P. nudicaule* are listed in Table 1.

Table 1. *Fusarium oxysporum* isolates from *Papaver nudicaule* used in this study.

Isolate	Host plant	Year of isolation	Place
759/11 IFR	*Papaver nudicaule*	2011	Northern Italy
811/11 IFR	*Papaver nudicaule*	2011	Northern Italy
658/11 IFR	*Papaver nudicaule*	2011	Northern Italy
782/11 IFR	*Papaver nudicaule*	2011	Northern Italy
889/11 IFR	*Papaver nudicaule*	2011	Northern Italy
Reis 1	*Papaver nudicaule*	2011	Northern Italy
Reis 2	*Papaver nudicaule*	2011	Northern Italy
Reis 4	*Papaver nudicaule*	2011	Northern Italy

Inoculum production for pathogenicity tests

The isolate used for the first report of the disease (Garibaldi *et al*., 2012) and three further isolates were tested in three pathogenicity tests. The isolates were grown for 10 days in potato dextrose broth (PDB, Sigma-Aldrich) on a shaker (90 rpm) at 22 ± 1°C with 12 h of fluorescent light per day. The culture suspension was filtered through one layer of cheese cloth and the concentration of conidia and mycelium fragments was determined with a haemocytometer. The suspension was then adjusted with deionized water to 1×10^7 Colony-Forming Units (CFU)/mL. Roots of two-month-old plants of *P. nudicaule* grown from seeds were dipped in the conidial suspension. In a first and a second pathogenicity test, 14 plants were inoculated with a single isolate; in a third test, three different isolates (3 plants/isolate) were tested. Roots of control plants were dipped in sterile water. Inoculated plants and controls were transplanted into a steamed potting mixture (sphagnum peat, perlite, pine bark, clay at 50:20:20:10) and maintained in a

greenhouse at temperatures ranging from 24 to 30°C. Plants were checked every 7 days and wilted plants were counted and removed.

DNA extraction

Total DNA was extracted using the NucleoSpin kit (Macherey Nagel GmbH and Co.), according to the manufacturer's instructions. One hundred mg of fresh mycelium were scraped from Petri dishes containing PDA and transferred into a 2 mL tube containing 400 µL of lysis buffer and two tungsten beads. Mycelium was homogenized by Qiagen tissue lyser for 3 min at 28 revolutions per min and the resulting solution was used for DNA extraction. 10 µL of Proteinase K solution (10 mg mL^{-1}) and 10 µL of RNAse A (12 mg mL^{-1}) were added to each tube. DNA concentration was measured with Nanodrop and stored at -20°C until use.

PCR amplification

Translation elongation factor 1-α *(EF-1α)*, the exopolygalacturonase genes *Pgx1* and *Pgx4* and the endopolygalacturonase genes *Pg1* and *Pg5* were amplified with the primers reported in Table 2. The PCR reaction was carried out in 20 µL volumes containing 10 ng of gDNA; 1 µL (10 mM stock) of each primer, 1 Unit of *Taq* DNA Polymerase (Qiagen), 2 µL of *Taq* DNA Polymerase buffer, 1 µL of dNTPs mix and 0.8 µL of $MgCl_2$ in a Gene Amp 9700 machine (Applied Biosystem) with the following program: 94°C for 5 min, 50 cycles with denaturation at 94°C for 1 min; annealing at 52°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. A negative control (no template DNA) was included in all experiments. Correct amplifications were verified by electrophoresis on 0.8% agarose gel (Agarose D-1 LOW EEO, Eppendorf). After purification with QIAquick PCR purification kit (Qiagen), PCR products were measured by Nanodrop and sent to BMR genomics sequencing service (http://www.bmr-genomics.it/). All sequences were deposited in GenBank with the accession numbers given in Table 3.

Alignment and phylogenetic analysis

Similarity searches (BLASTN, default parameters) were performed in GenBank for all sequences. The sequences obtained were used for a CLUSTALW multiple sequence alignment through MEGA5 software (Tamura *et al.*, 2011) with default parameters. Manual corrections were performed for each alignment to trim regions outside and discard incomplete sequences. Phylogenetic trees were generated for each genomic region using MEGA5 software with Neighbor-joining using traditional search, with standard bootstrapping (1,000 replicates). The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. In each analysis the sequences derived from different *F. oxysporum formae speciales* obtained from GenBank were included.

Table 2. Primers used to amplify *EF-1α* and polygalacturonase genes.

Gene	Primer	Nucleotide Sequences (5'→3')	Source
Tef-1α	Ef1	ATGGGTAAGGAAGACAAGAC	O'Donnell *et al.*, 1998
	Ef2	GGAAGTACCAGTGATCATGTT	
Pg1	endoF	CCAGAGTGCCGATACCGATT	Hirano and Arie, 2009
	endoR2	GCTTAGYGAACAKGGAGTG	
Pg5	PG2F	AGATGCAAGGCCGATGATGT	Hirano and Arie, 2009
	PG2R	TCCATGTACTTCTCCTCACC	
Pgx1	PgxF	TCGTGGGGTAAAGCGTGGT	Hirano and Arie, 2009
	PgxR	TTACTATAGGTCGATCAGCC	
Pgx4	exoF2	TTACTGTCCACGAATGAGAAG	Hirano and Arie, 2009
	exoR	ACCCCAACCCCCCTCATCT	

Table 3. Accession numbers of *EF-1α*, *Pg1*, *Pg5*, *Pgx1* and *Pgx4* genes deposited in GenBank and obtained from *Fusarium oxysporum* isolates from *Papaver nudicaule*.

Isolate	GenBank Accession Number				
	EF-1alpha	Pg1	Pg5	Pgx1	Pgx4
759/11IFR	KF301629	KC900500	KC900508	KC880130	KC880138
811/11IFR	KF301630	KC900501	KC900512	KC880131	KC880139
658/11IFR	KF301631	KC900502	KC900513	KC880132	KC880140
782/11IFR	KF301632	KC900503	KC900511	KC880133	KC880141
889/11IFR	KF301633	KC900504	KC900514	KC880134	KC880142
Reis1	KF301634	KC900505	KC900509	KC880135	KC880143
Reis2	KF301635	KC900506	KC900515	KC880136	KC880144
Reis4	KF301636	KC900507	KC900510	KC880137	KC880145

Results

Pathogenicity tests

All inoculated plants (100% over 14 inoculated plants in Test 1 and 2, and over 3 plants in Test 3) wilted and died, showing typical symptoms of Fusarium wilt about ten days after inoculation. *Fusarium oxysporum* was consistently reisolated from infected plants and preserved as Reis. 1, 2, 4 (Table 1).

Phylogenetic analysis

Amplification of the *EF-1α*, *Pg1*, *Pg5*, *Pgx1* and *Pgx4* genes resulted in products with 720, 1,560, 1,800, 1,800 and 1,400 bp. In the phylogenetic analysis the isolates derived from *P. nudicaule* always clustered in a unique clade separate from other *formae speciales* of *F. oxysporum* (Figures 1–5). For most genes the bootstrap values for these clades were high with more than 85% for *EF-1α* (98%), *Pg1* (87%) and *Pgx1* (87%).

Discussion

Polygalacturonases are involved in the first step of the pathogen-plant interaction. Phylogenetic analysis based on sequences from *EF-1α* together with four polygalacturonase genes was useful in previous phylogenetic studies in *Fusarium* (Hirano and Arie, 2009). Our results show a cluster of isolates from *P. nudicaule* separated from all other known *F. oxysporum formae speciales* (Figures 1–5). Infection of *P. nudicaule* by *F. oxysporum* was first reported by Garibaldi *et al*. (2012). The present study corroborates the introduction of a new forma specialis; isolate 759/11 IRF was registered in MycoBank with the accession number MB810991.

Acknowledgements

This work was supported by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 261752, PLANTFOODSEC "Plant and Food Biosecurity, Network of Excellence".

Literature cited

Armstrong G.M. and J.K. Armstrong, 1981. *Formae speciales* and races of *Fusarium oxysporum* causing wilt diseases. In *Fusarium*: Diseases, Biology and Taxonomy, R. Cook, (ed.), University Park, PA: Penn State University Press, pp. 391–399.

Baayen R.P., K. O'Donnell, P.J. Bonants, E. Cigelnik, L.P. Kroon, E.J. Roebroeck and C. Waalwijk, 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic *formae speciales* causing wilt and rot disease. *Phytopathology* 90, 891–900.

Di Pietro A., M.P. Madrid, Z. Caracuel, J. Delgado-Jarana and M.I.G. Roncero, 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Molecular Plant Pathology* 4, 315–325.

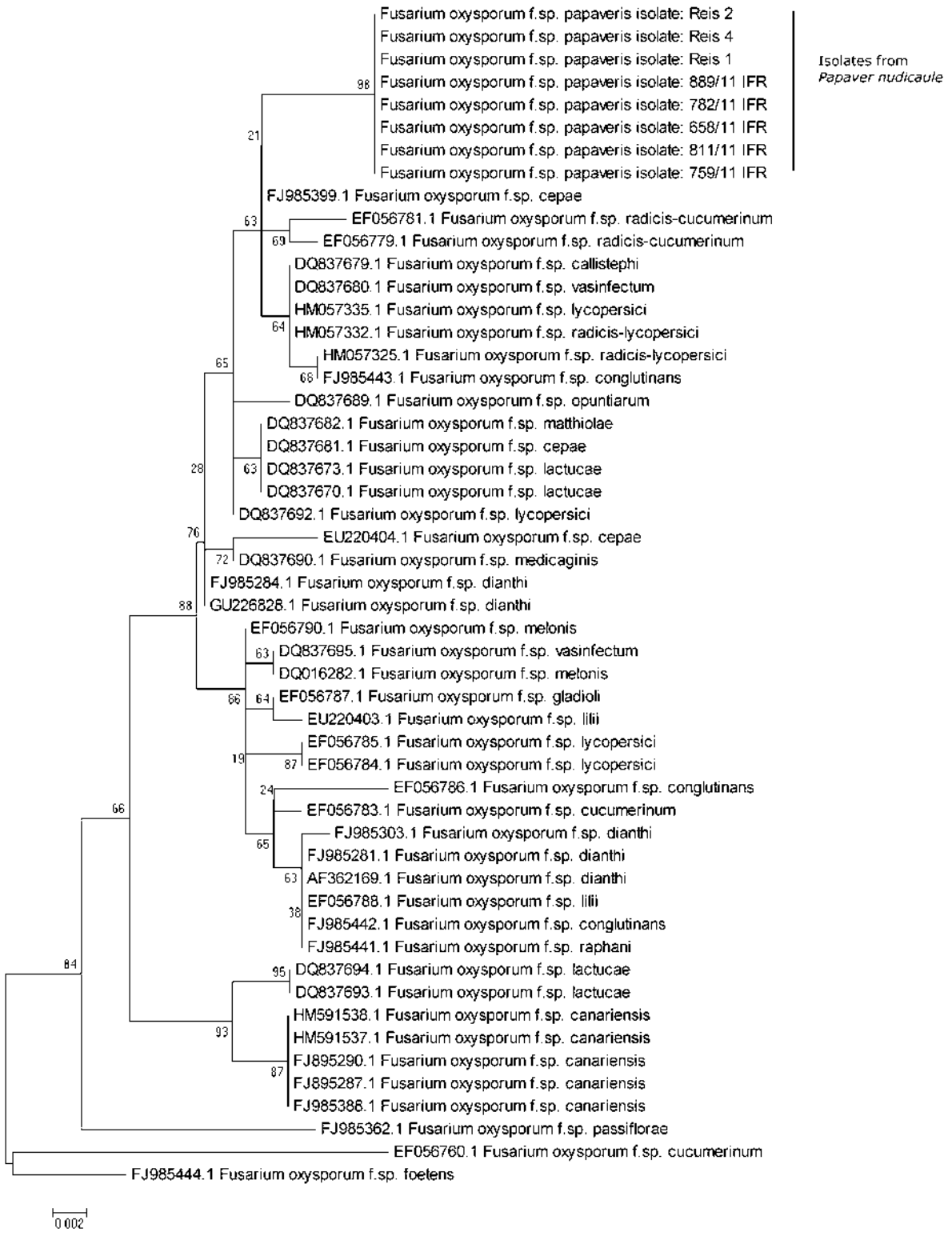

Figure 1. Phylogenetic tree based on *EF-1α* gene sequences built by Mega5 software with Neighbor joining method; bootstrap values from 1000 replicates are shown at the nodes.

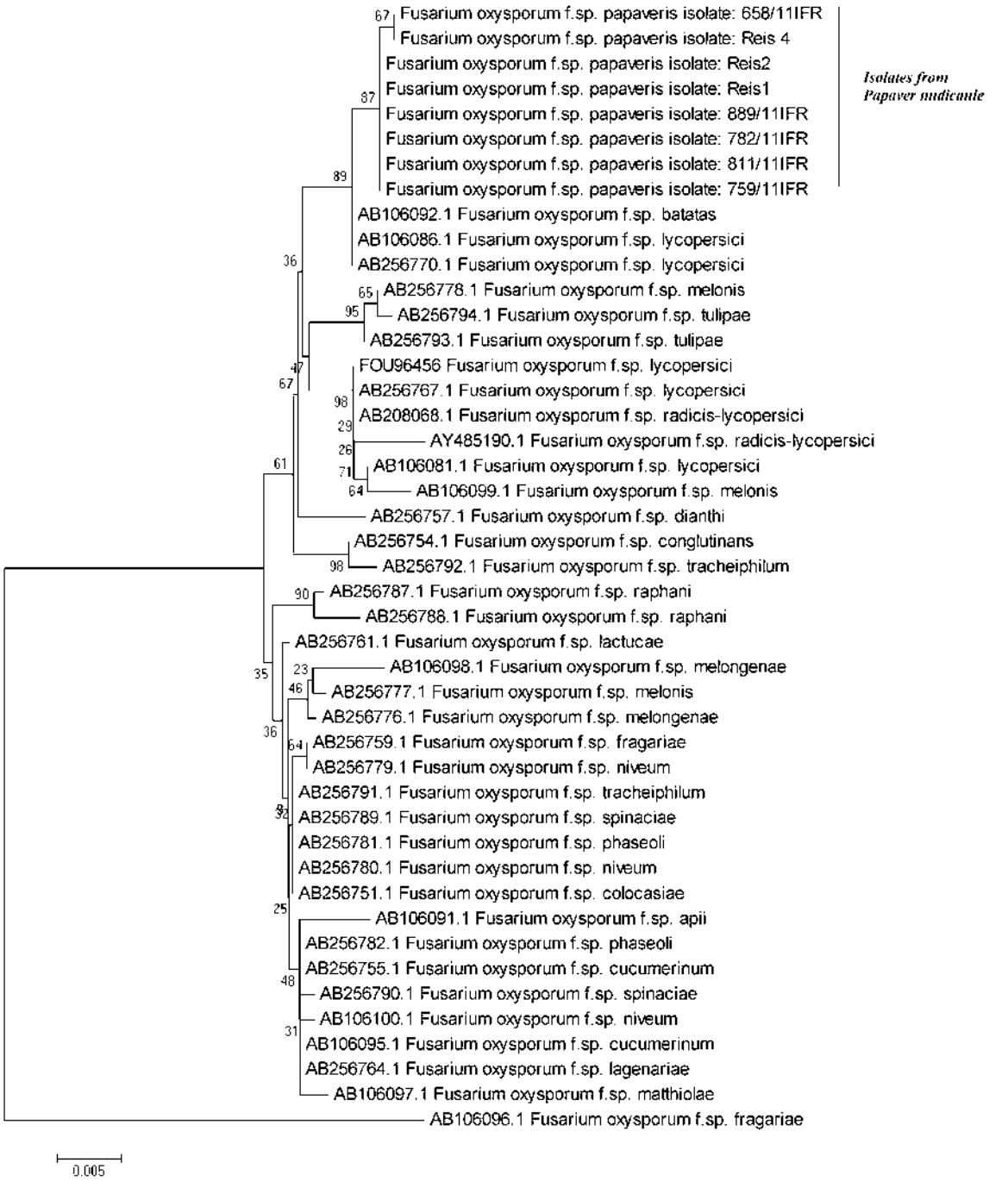

Figure 2. Phylogenetic tree based on *Pg1* gene sequences built by Mega5 software with Neighbor joining method; bootstrap values from 1000 replicates are shown at the nodes.

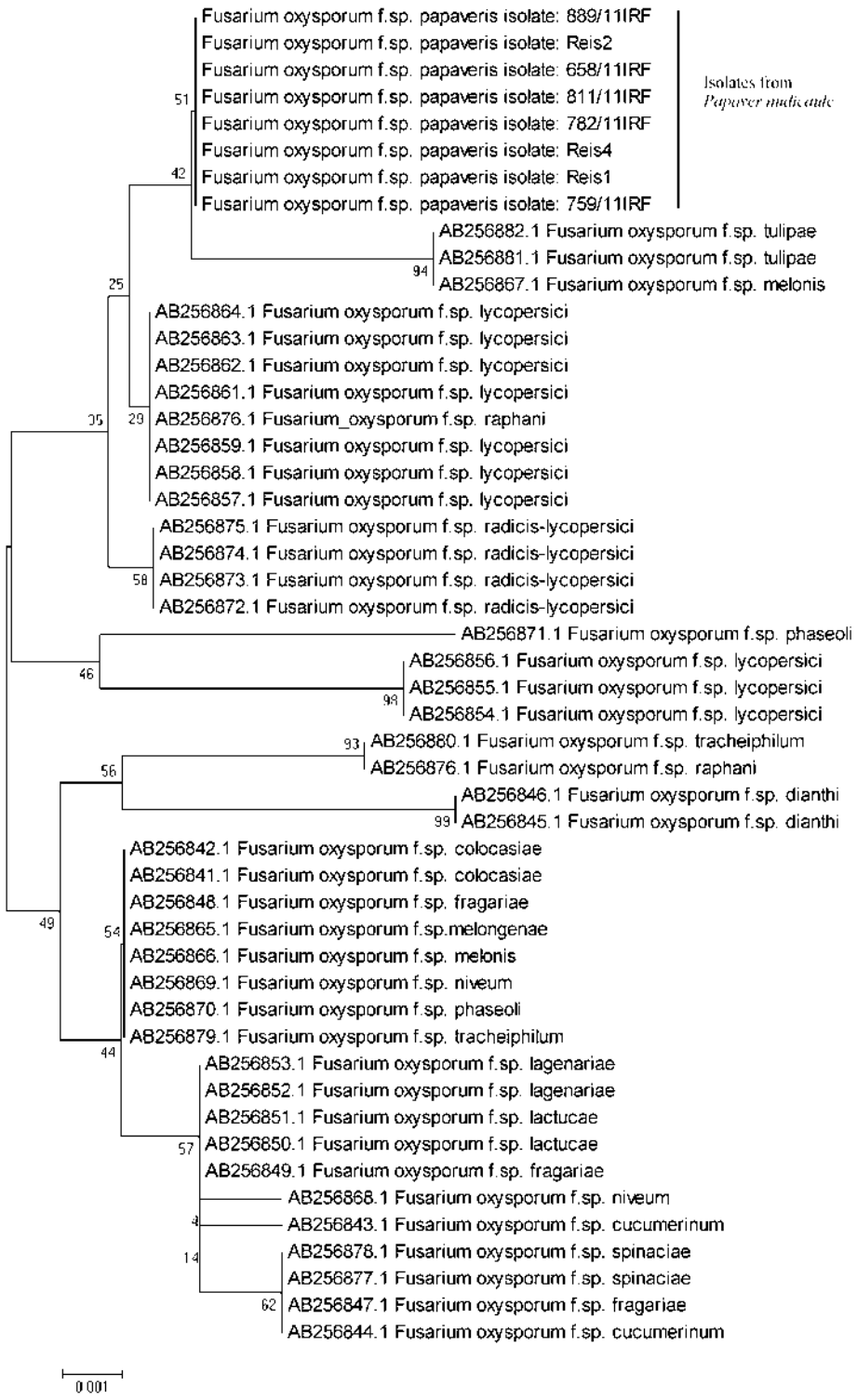

Figure 3. Phylogenetic tree based on *Pg5* gene sequences built by Mega5 software with Neighbor joining method; bootstrap values from 1000 replicates are shown at the nodes.

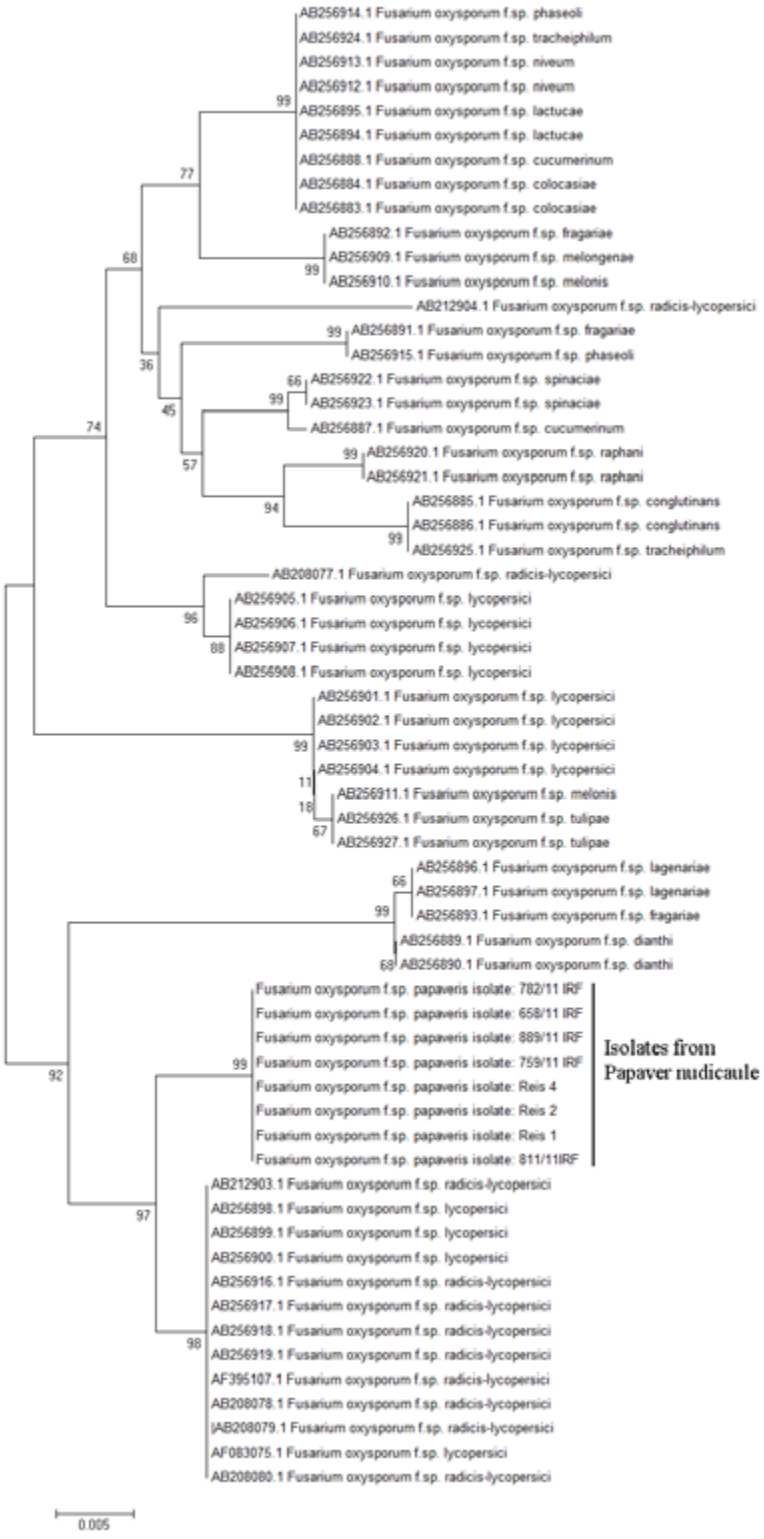

Figure 4. Phylogenetic tree based on *Pgx1* gene sequences built by Mega5 software with Neighbor joining method; bootstrap values from 1000 replicates are shown at the nodes.

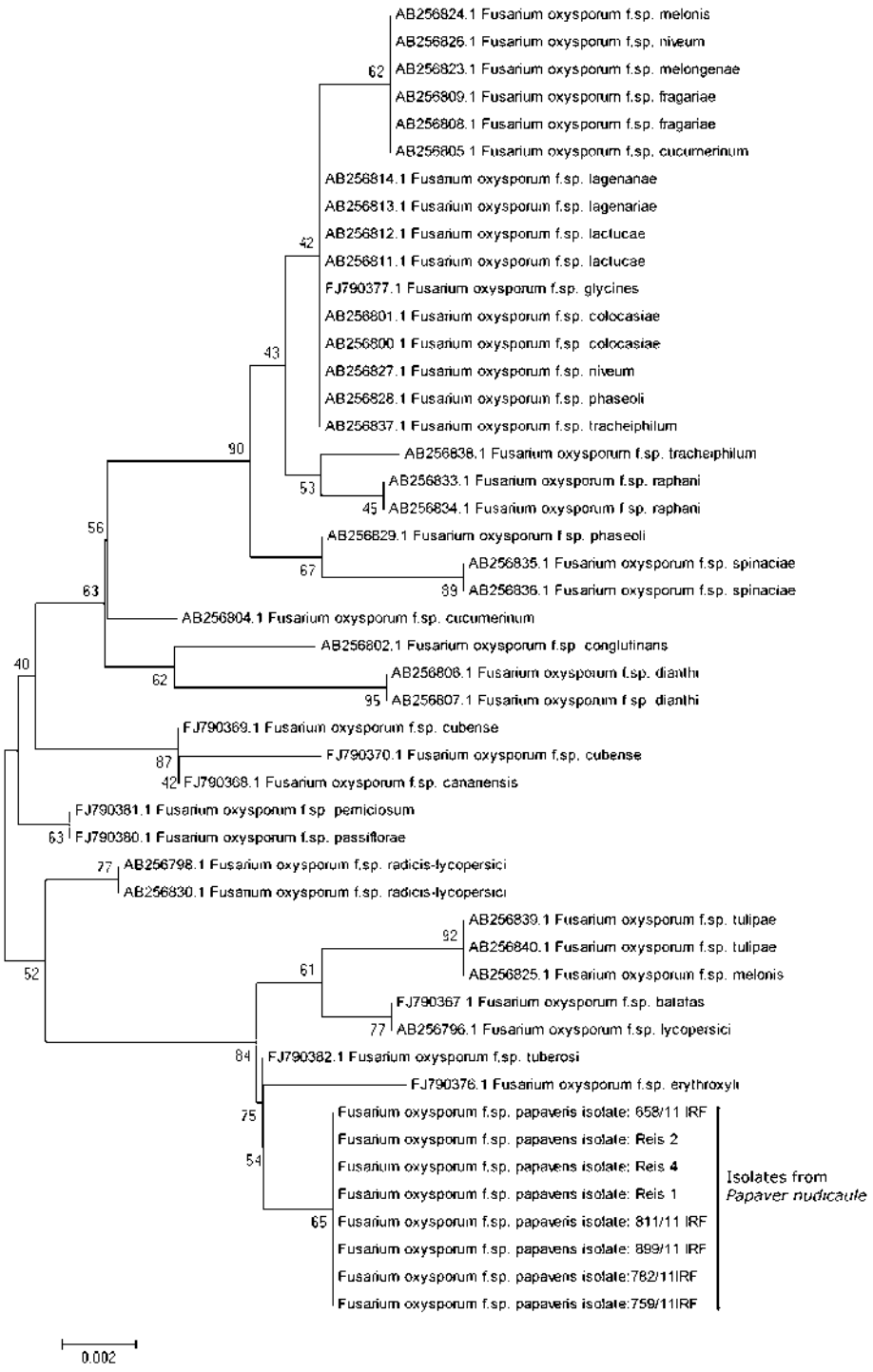

Figure 5. Phylogenetic tree based on *Pgx4* gene sequences built by Mega5 software with Neighbor joining method; bootstrap values from 1000 replicates are shown at the nodes.

Friesen T.L., E.H. Stukenbrock, Z.H. Liu, S. Meinhardt, H. Ling, J.D. Faris, J.B. Rasmussen, P.S. Solomon, B.A. McDonald and R.P. Oliver, 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics* 38, 953–956.

Garibaldi A. and M.L. Gullino, 1985. Wilt of *Ranunculus asiaticus* caused by *Fusarium oxysporum* f. sp. *ranunculi*, *forma specialis* nova. *Phytopathologia Mediterranea* 24, 213–214.

Garibaldi A., P. Martini, L. Repetto, M. Odasso, D. Bertetti, A. Poli and M.L. Gullino, 2012. First Report of *Fusarium oxysporum* causing wilt on Iceland Poppy (*Papaver nudicaule*) in Italy. *Plant Disease* 96, 1823.

Hirano Y. and T. Arie, 2006. PCR-based differentiation of *Fusarium oxysporum* ff. spp. *lycopersici* and *radicis-lycopersici* and races of *F. oxysporum* f. sp. *lycopersici*. *Journal of General Plant Pathology* 72,273–283.

Hirano Y. and T. Arie, 2009. Variation and phylogeny of *Fusarium oxysporum* isolates based on nucleotide sequences of polygalacturonase genes. *Microbes and Environments* 24, 113–120.

Joosten M., T.J. Cozijnsen and P. Dewit, 1994. Host-resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature* 367, 384–386.

Kirk P., P. Cannon, J. Stalpers, 2008. Dictionary of the Fungi.10th edition CAB International, Wallingford.

Komada H. 1975 Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soils. *Review of Plant Protection Research* 8, 114–125.

Leslie J.F. 2012. Genetics and *Fusarium oxysporum.* In Gullino ML, J. Katan, A. Garibaldi (ed.), Fusarium wilts of greenhouse vegetable and ornamental crops. APS Press, St.Paul, MN. pp. 39–47.

Lutzoni F., F. Kauff, C.J. Cox, D. McLaughlin, G. Celio, B. Dentinger, M. Padamsee, D. Hibbett, T.Y. James, E. Baloch, M. Grube, V. Reeb, V. Hofstetter, C. Schoch, A.E. Arnold, J. Miadlikowska, J. Spatafora, D. Johnson, S. Hambleton, M. Crockett, R. Shoemaker, G.H. Sung, R. Lücking, T. Lumbsch, K. O'Donnell, M. Binder, P. Diederich, D. Ertz, C. Gueidan, K. Hansen, R.C. Harris, K. Hosaka, Y.W. Lim, B. Matheny, H. Nishida, D. Pfister, J. Rogers, A. Rossman, I. Schmitt, H. Sipman, J. Stone, J. Sugiyama, R. Yahr and R. Vilgalys, 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany* 91, 1446–1480.

O'Donnell K., H.C. Kistler, E. Cigelnik and R.C. Ploetz, 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences* 95, 2044–2049.

O'Donnell K. and E. Cigelnik, 1999. A DNA sequence-based phylogenetic structure for the *Fusarium oxysporum* complex. *Phytoparasitica* 27, 69–70.

O'Donnell K., C. Gueidan, S. Sink, P.R. Johnston, P.W. Crous, A. Glenn, R. Riley, N.C. Zitomer, P. Colyer, C. Waalwijk, T. Lee, A. Moretti, S. Kang, H.S. Kim, D.M. Geiser, J.H. Juba, R.P. Baayen, M.G. Cromey, S. Bithell, D.A. Sutton, K. Skovgaard, R. Ploetz, K. H. Corby, M. Elliott, M. Davis and B.A. Sarver, 2009. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genetics and Biology* 46, 936–948.

Ortu G., D. Bertetti, M.L. Gullino and A. Garibaldi, 2013. A new *forma specialis* of *Fusarium oxysporum* on *Crassula ovata*. *Journal of Plant Pathology* 95, 33–39.

Preininger V. 1986. In The Alkaloids (Chemistry and Pharmacology). Chemotaxonomy of Papaveraceae and Fumariaceae, A. Brossi (ed.), Academic Press Inc., Orlando, San Diego, New York, Austin, Boston, London, Sydney, Tokyo and Toronto 29, 1–98.

Recorbet G., C. Steinberg, C. Olivain, V. Edel, S. Trouvelot, E. Dumas-Gaudot, S. Gianinazzi and C. Alabouvette, 2003. Wanted: pathogenesis-related marker molecules for *Fusarium oxysporum*. *New Phytologist* 159, 73–92.

Rep M., M. Meijer, P.M. Houterman, H.C. Van Der Does and B.J.C. Cornelissen, 2005. *Fusarium oxysporum* evades I-3-mediated resistance without altering the matching avirulence gene. *Molecular Plant Microbe Interaction* 18, 15–23.

Rep M., H.C. van der Does, M. Meijer, R. van Wijk, P.M. Houterman, H.L. Dekker, C.G. de Koster and B.J.C. Cornelissen 2004. A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Molecular Microbiology* 53, 1373–1383.

Schilling A.G., E.M. Moller, H.H. Geiger, 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. *Phytopathology* 86, 515–522.

Soltis P.S., D.E. Soltis and M.W. Chase 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* 402, 402–404.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution. *Molecular Biology and Evol*ution 28: 2731–2739

Van der Does H.C. and M. Rep, 2007. Virulence genes and the evolution of host specificity in plant-pathogenic fungi. *Molecular Plant Microbe Interaction* 20, 1175–1182.

Věžník F., E. Táborská, H. Bochořáková, F. Tureček, V. Hanuš and J. Slavík, 1987. Alkaloids of *Papaver nudicaule* subsp. *xanthopetalum* (Trautv.) Fedde and *P. nudicaule* subsp. *album* (Regel) Fedde from the section *Scapiflora* Reichb. *Collection of Czechoslovak Chemical Community* 52, 1634–1640.

Accepted for publication: December 9, 2014
Published online: April 14, 2015